we do not know its identity with certainty, its electrophoretic mobilities appear consistent with its being the 10 000-dalton acidic subunit that has been reported by Moreira et al. (1979). Spot Z diffused rather readily from our gels and was erratic in amount.

Moreira et al. (1979) reported that with the exception of acidic subunit A4, the acidic subunits of glycinin are joined to basic subunits by disulfide linkages. A4 had the lowest p*I* among the acid subunits (Moreira et al., 1981). We have found two spots that appear to be acidic subunits of glycinin that are preferentially enriched in whey and, to a lesser extent, in the fraction not coagulated by heating. Those spots are the most acidic of the glycinin subunits. Thus, we can tentatively identify our spots "X" with the A4 subunit of Moreira et al. (1979). If that identification is correct, it suggests that the absence of a disulfide linkage between the A4 subunits and the basic subunits could contribute to the enrichment of the A4 subunits in the whey proteins.

ACKNOWLEDGMENT

We thank Dr. N. C. Nielsen of Purdue University for samples of glycinin and conglycinin that we used in the early stages of this work.

Registry No. Kunitz trypsin inhibitor, 9088-41-9.

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Received for review August 5, 1982. Revised manuscript received February 7, 1983. Accepted April 22, 1983. Supported by National Institutes of Health Research Career Development Award CA-00425. Contribution No. 82-505-j, Kansas Agricultural Experiment Station.

Amidation or Esterification of Bovine β-Lactoglobulin To Form Positively Charged Proteins

Nina L. Mattarella,[1] Lawrence K. Creamer,[2] and Thomas Richardson*

Bovine β-lactoglobulin was amidated or esterified to yield modified proteins with 55–83% of the free carboxyl groups blocked and with mean isoionic points of 7–10 compared to 5.2 for the native protein. Upon gel electrophoresis at acidic pH values or in sodium dodecyl sulfate, the amidated protein was polydisperse and contained small quantities of dimer, whereas esterified proteins were less polydisperse and not dimerized. A more random structure as a result of amidation or esterification was evident from examination of the modified proteins using circular dichroism.

Whey, the liquid remaining after the process of cheese making, contains most of the salts, lactose, and water-soluble proteins of the milk. The world production of cheese whey has been estimated to be approximately 72 000 million kg (Kosikoski, 1979), much of which is disposed of without being utilized. Undenatured whey proteins can be separated from the liquid by ultrafiltration, ion-exchange chromatography, or gel filtration chromatography and are marketed as whey protein concentrates. Besides having excellent nutritional quality, the undenatured whey proteins are soluble over a wide pH range, have good emulsification capacity and whipping ability, and form gels when subjected to heat treatments under proper conditions (Marshall, 1979).

With the advent of ultrafiltration, more whey processors are marketing whey protein concentrates. The result is an excess of the products on the market with an attendant decline of price and, therefore, profitability. This factor along with competition from plant protein concentrates necessitates additional research for the development of new markets for whey proteins.

Alteration or extension of the functional properties of whey proteins may result by changing the net charge of the proteins from negative to positive at pH 7. It should be possible to prepare positively charged proteins by

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0021-8561/83/1431-0968$01.50/0

adding positive or subtracting negative charges. Thus, a positively charged protein can be prepared by blocking ω-carboxyl groups in proteins. While studying exposed carboxyl groups in proteins, Lewis and Shafer (1973) converted them to carboximido residues using a carbodiimide-mediated condensation with ammonium ion. Esterification with alcoholic HCl has also proven useful for blocking exposed carboxyl groups in proteins (Wilcox, 1972).

This study was undertaken to explore the effects of amidation or esterification have on the major whey protein, β-lactoglobulin, as a preliminary to modifications of whey protein concentrate.

EXPERIMENTAL SECTION

Amidation. Purified β-lactoglobulin (3× crystallized, Sigma, St. Louis, MO) was amidated by a modification of the method of Lewis and Shafer (1973). A solution of β-lactoglobulin (0.5%) in 5.5 M ammonium chloride was adjusted to pH 4.75 with dilute HCl. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) (Aldrich, Milwaukee, WI) was added (0.1 mmol of EDC/mL of protein solution), and the pH of the protein solution was maintained at 4.75 by addition of dilute HCl or dilute NaOH. The reaction was stopped after 5–180 min either by dialysis of the protein solution against 0.001 M HCl or by passage of the protein solution through a Sephadex G-25 column by using 0.001 M HCl to elute the protein. The modified protein was freeze-dried and stored at 0 °C.

Esterification. Esters of β-lactoglobulin were prepared by using a modification of the procedure described by Fraenkel-Conrat and Olcott (1945). Purified β-lactoglobulin was suspended in the cold alcohol (methanol, ethanol, propanol, 1-butanol, or *n*-amyl alcohol) to give a 1% suspension. While the protein–alcohol suspension was stirred, concentrated HCl was slowly added to make the suspension 0.07 M in HCl. Each mixture was stirred at 4 °C for various periods of time and was then diluted 1:1 with cold deionized water, dialyzed against 0.001 M HCl, and freeze-dried.

Electrophoresis and Isoionic Points. Electrophoresis in sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gels followed the general procedure of Laemmli (1970). Modified β-lactoglobulin was also examined by discontinuous, polyacrylamide gel electrophoresis at an acidic pH (Reisfeld et al., 1962).

Ten to fifteen milligrams of protein was dissolved in 10 mL of deionzied water with stirring. One gram of mixed-bed ion-exchange resin (AG501-X8D, Bio-Rad, Richmond, CA) was added to the protein solution and the pH monitored until it became constant. The isoionic point of each protein was considered to be this pH, and proteins of known isoionic points were used as standards (Ho and Waugh, 1965).

Titration Curve. β-Lactoglobulin in 0.15 M KCl was titrated with standard KOH and HCl at 25 °C.

The KOH solution was prepared by dissolving KOH to approximately 5 M and precipitating carbonate with $Ba(OH)_2$. After centrifugation, the supernatant solution was diluted to approximately 1 M and standardized against potassium hydrogen phthalate. The resultant KOH solution was used to standardize the HCl solution.

A Radiometer Model 26 pH meter fitted with a Corning combination electrode, No. 476050, was used to record pH changes. The protein solution (5 mL), a KCl solution (5 mL) and deionized distilled water (10 mL) were mixed, and a stream of nitrogen saturated with water vapor was passed over the solution. The titrant was added (usually 10 μL at a time) to the stirred solution, and readings were taken when the pH of the solution became stationary. The protein titration data were corrected by subtracting the appropriate titrations of blank solutions.

Table I. Isoelectric Points Compared with Isoionic Points of Various Proteins

protein	isoelectric point	apparent isoionic point[a]
bovine serum albumin	4.7, 4.9[b]	5.0
trypsin inhibitor (soybean)	4.5[b]	4.5
lysozyme (egg white)	11.00[c]	10.3
ribonuclease (bovine)	9.3[b]	9.3
myoglobin (sperm whale)	8.18, 7.67[d]	7.9
β-lactoglobulin (bovine)	5.2, 5.3[d]	5.2

[a] Determined by the method of Ho and Waugh (1965). [b] Malamud and Drysdale (1978). [c] Alderton et al. (1945). [d] Radola (1976).

Circular Dichroism Measurements. A Jasco (Japan Spectroscopic Co., Tokyo) Model J-41C spectropolarimeter with a spectral bandwidth of 2 nm, a scanning rate of 1.0 nm/min, and a time constant of 16 s was used to record circular dichroism spectra. Protein solutions of 0.1 mg/mL in a 1-mm cell were scanned between 190 and 250 nm. Molar ellipticity per residue, [θ], in deg cm^2 $dmol^{-1}$ was calculated from

$$[\theta] = 100dMn^{-1}C^{-1} \quad (1)$$

where d is the rotation in deg/1.0-cm path length of solution, M is the molecular weight of the protein [assumed to be 18362 for β-lactoglobulin and modified β-lactoglobulin (Whitney et al., 1976)], n is the number of residues in the protein chain [162 for β-lactoglobulin (Braunitzer et al., 1973)], and C is the concentration of protein in mg/mL. For scans between 250 and 340 nm a 1-cm cell was used and protein solutions contained 0.6 mg/mL. The molecular circular dichroism, $\Delta\epsilon$, was calculated from

$$\Delta\epsilon = 0.0304dMC \quad (2)$$

where the terms have the same meaning as that for the previous equation.

A solution of *d*-camphor-10-sulfonic acid (60 mg/mL), which has a positive circular dichroism, $\Delta\epsilon$, of 2.20 (a molar ellipticity of 7260 deg cm^2 $decimol^{-1}$) at 290.5 nm (Cassim and Yang, 1969), was used to calibrate the spectropolarimeter.

RESULTS AND DISCUSSION

Isoelectric points, determined as the pH at which a protein has zero mobility in an electric field, and isoionic points, the pH of a protein solution with no ions present except the hydrogen and hydroxyl ions produced by the dissociation of water and the protein, are generally very close in value. Published isoelectric points of various proteins were compared with their isoionic points determined simply by adding a mixed-bed ion-exchange resin to the protein solution (Table I). Except for lysozyme, the values are within 0.3 pH unit of literature values, and the mixed-bed resin method for determining isoionic points is simple, rapid, and excellent for preliminary measurements. The discrepancy with lysozyme may be related to an aggregation of the protein that was observed 5 min after addition of the mixed-bed resin.

The average isoionic points of amidated β-lactoglobulin determined after dialysis of solutions obtained 5–180 min following addition of EDC differed only slightly. Isoionic points varied between 9 and 10 compared to 5.2 for native β-lactoglobulin. The longer reaction times tended to yield proteins with higher isoionic points. Continued reaction

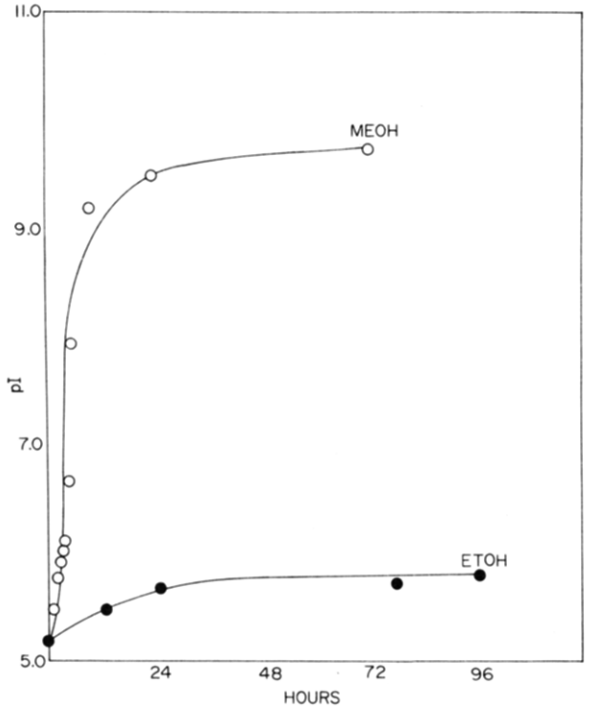

Figure 1. Rate of esterification as measured by the increase in isoionic points (p*I*) of the derivatives. $n = 3$; reported as average values.

during dialysis may have accounted for the small differences in isoionic points. However, when portions of protein solution taken after 5- and 180-min reaction times were rapidly purified by gel filtration chromatography on Sephadex G-25, virtually no differences were observed in the extent of amidation. Thus, at pH 4.75 and in 5.5 M NH_4Cl, the carbodiimide-mediated conversion of exposed carboxyl groups of aspartyl and glutamyl residues in β-lactoglobulin to asparaginyl and glutaminyl residues is essentially complete in 5 min. Consequently, β-lactoglobulin was subsequently amidated by using a 5-min reaction time.

Esters of the carboxyl groups of β-lactoglobulin were prepared by using methanol or ethanol. Esterification of β-lactoglobulin by methanol, as inferred from the isoionic point of the modified protein, proceeded much more quickly than esterification with ethanol and was sufficiently complete after 24 h at 4 °C (Figure 1). Although these early esterifications indicate an inordinate amount of time would be required to attain a p*I* of 7 in the ethyl esterification, subsequent esterification rates were increased by using dried reagents and conditions. Esterification by propyl, butyl, or amyl alcohols after 1 week was not evident since there was no change in isoionic points of the treated proteins. Fraenkel-Conrat and Olcott (1945) reported esterification of polyglutamic acid with methanol–HCl proceeded much more rapidly than with ethanol–HCl. These authors also reported that esterification of proteins with higher primary alcohols occurs but that the rate of esterification is slower and the extent, therefore, is progressively less.

Electrophoresis in polyacrylamide gels under acidic conditions of representative samples of amidated or esterified β-lactoglobulin yielded patterns shown in Figures 2 and 3. Lysozyme was used as a standard, positively charged protein. The amidated β-lactoglobulin gel pattern (Figure 2) was smeared, which indicated a polydisperse distribution of positively charged species. The polydispersity may relate to the two-step reaction of a protein with

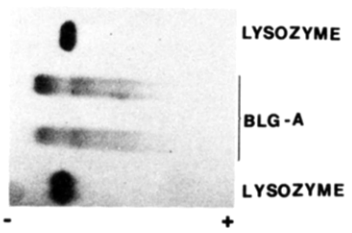

Figure 2. Electrophoretic pattern of amidated β-lactoglobulin (BLG-A) and lysozyme in an acidic polyacrylamide gel.

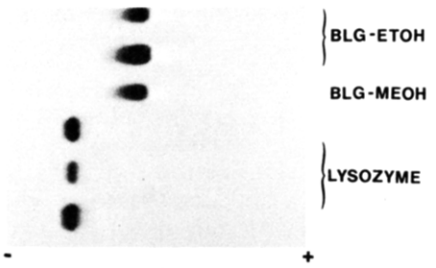

Figure 3. Electrophoretic pattern of ethyl (EtOH)- and methyl (MeOH)-esterified β-lactoglobulins and lysozyme, in an acidic polyacrylamide gel.

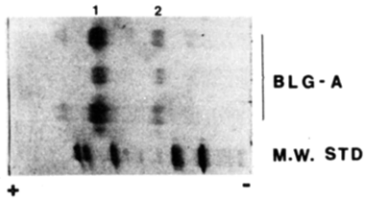

Figure 4. Electrophoretic pattern of amidated β-lactoglobulins (BLG-A) compared to those of standard proteins in an $NaDodSO_4$–polyacrylamide gel. The molecular weight of BLG-A1 is about 18 000, whereas that of BLG-A2 is about 36 000. Standard proteins are lysozyme, 14 300, β-lactoglobulin, 18 400, trypsinogen, PMSF treated, 24 000, albumin, egg, 45 000, and albumin, bovine, 66 000.

a water-soluble carbodiimide which appears to involve the formation of an intermediate *O*-acylisourea. This intermediate may hydrolyze or condense with amines and other nucleophiles (Khorana, 1953). The limited nature and extent of cross-linking may reflect the high concentration of ammonium chloride in the reaction mixture. By comparison, bands of the esterified proteins (Figure 3) were spread only slightly. The apparent, anomalous migrations of amidated β-lactoglobulin compared to lysozyme (Figure 2) and the methyl compared to the ethyl-esterified proteins (Figure 3) are difficult to explain. The observed anomalies may reflect changes in protein conformations as a result of derivatization which could alter their size and shape as well as charge. In the case of the methyl- and ethyl-esterified proteins (Figure 3), the methyl esters may be preferentially hydrolyzed under the acidic conditions of electrophoresis. Upon $NaDodSO_4$–polyacrylamide gel electrophoresis, one preparation of the amidated β-lactoglobulin contained a slight amount of dimer and trimer (Figure 4). The methyl and ethyl esters of β-lactoglobulin, however, were free of dimers as indicated from the $NaDodSO_4$–polyacrylamide gel electrophoretic patterns (Figure 5), but some fragmentation of the proteins was evidenced by lower molecular weight species.

The titration curves in the acidic range of the modified proteins compared to that of the native β-lactoglobulin indicated 78% of the carboxyl groups were amidated. On the other hand, approximately 55% of the carboxyl groups in the ethyl-esterified β-lactoglobulin and 83% of the

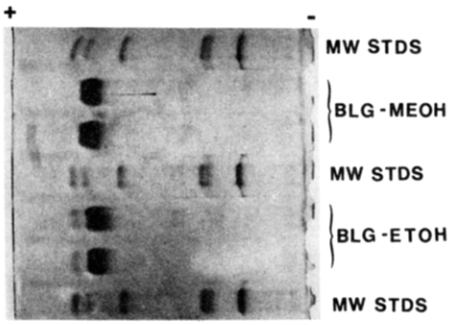

Figure 5. Electrophoretic pattern of ethyl (EtOH)- and methyl (MeOH)-esterified β-lactoglobulins compared to those of standard proteins in a $NaDodSO_4$–polyacrylamide gel. Molecular weights of the esterified β-lactoglobulins are about 18000.

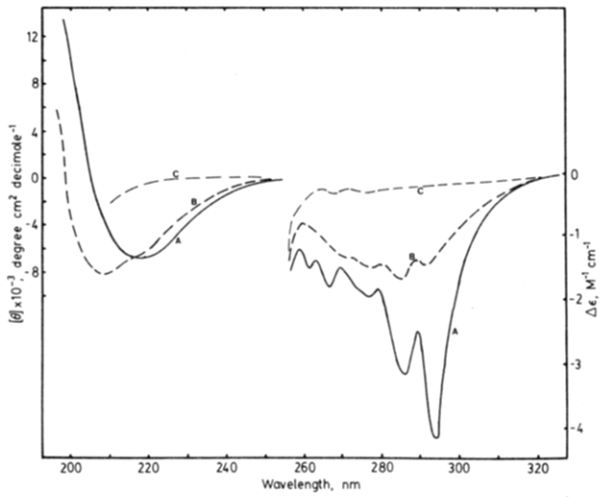

Figure 6. CD spectra of β-lactoglobulin (A), amidated β-lactoglobulin (B), and β-lactoglobulin in 5 M guanidine hydrochloride (Gdn·HCl) (C).

carboxyl groups in the methyl-esterified β-lactoglobulin were blocked. Since there are 27 aspartyl and glutamyl residues in β-lactoglobulin (Whitney et al., 1976), an average of 21 acidic residues were modified in the amidated protein, 15 in the ethyl-esterified protein and 22 in the methyl-esterified β-lactoglobulin. Isoionic points as estimated by using the mixed-bed resin procedure were 10, 7.2, and 9.8, respectively.

It was possible, therefore, to amidate or esterify β-lactoglobulin under mild conditions, forming positively charged proteins by modifying the carboxyl groups. The carbodiimide-mediated condensation of ammonia with carboxyl groups proceeded very rapidly, but some carboxyl residues apparently were not sufficiently exposed to react with the carbodiimide or ammonium ion. The reaction yielded a polydisperse protein with some dimerization due to intermolecular condensation. Esterification also was not complete but yielded a less disperse protein with no dimerization. The isoionic points of modified β-lactoglobulins increased from 5.2 for native protein to 7–10 for the various modifications, indicating possibilities for controlling the mean net charge of proteins.

The circular dichroism spectra of native or modified β-lactoglobulins in phosphate buffer or guanidine hydrochloride (Gdn·HCl) solutions were measured in the range of 190–350 nm (Figures 6 and 7). Native β-lactoglobulin gave a spectrum essentially the same as that reported earlier (Timasheff et al., 1967) although the molar ellipticities were slightly greater in our study. The CD spectrum is similar to that of a protein with a predominantly β-sheet structure. Application of various structure analysis

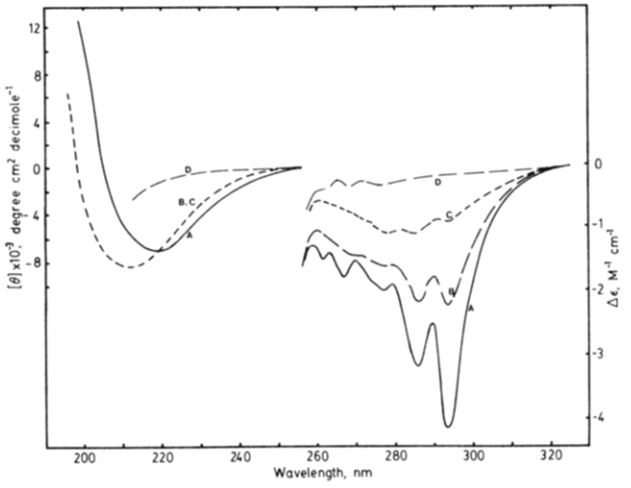

Figure 7. CD spectra of β-lactoglobulin (A), ethyl-esterified β-lactoglobulin (B), methyl-esterified β-lactoglobulin (C), and β-lactoglobulin in 5 M guanidine hydrochloride (D).

procedures gave somewhat different results depending upon the method of analysis. The most sophisticated techniques involve computer analysis systems where the molar ellipticities at numerous, different wavelengths are compared, by least squares, with standard spectra. Solution of resulting simultaneous equations gives an indication of the α-helical, β-sheet, and aperiodic structural content of the protein under study. Such a computer program [Bloom and Mann (1978), as derived from Chen et al. (1972, 1974)] indicated the structure of β-lactoglobulin was about 16% α-helical, 54% β-sheet, and 30% aperiodic whereas that of the amidated β-lactoglobulin was about 20% α-helical, 29% β-sheet, and 51% aperiodic. The more sophisticated approach of Provencher and Gloeckner (1981) gave essentially the same results (Malcom and Creamer, unpublished data). The use of a different computer program (Siegel et al., 1980) gave 18% α-helical structure for the native protein and 16% for the amidated protein.

The main feature of random coil or aperiodic structure is a deep trough near 200 nm, whereas both α-helical and β-sheet structures have peaks near 195 nm and troughs in the 210–230-nm region. Inspection of the spectra reveals that there was a definite decrease in the amount of ordered structure in the modified β-lactoglobulins compared to the native protein.

The near-UV CD spectra are indicative of the environment around the aromatic chromophores. If an aromatic chromophore is close to another and asymmetry is involved, then the asymmetric energy transfer between the chromophores results in an enhanced ellipticity and a positive or negative peak in the CD spectra. Comparison of the spectrum of the native protein with that of the completely disordered protein in 5 M Gdn·HCl indicates a considerable enhancement of negative ellipticity for the native protein probably caused by asymmetric energy transfer. Although the assignment of the negative peaks is not straightforward, those at 293 and 286 nm are undoubtedly caused by one or both of the tryptophan residues in a stable nonrandom configuration. The minor troughs near 262 and 270 nm (also observable in the Gdn·HCl spectrum) are probably caused by order in phenylalanine residues (Strickland, 1974). These troughs disappeared upon amidation or esterification of β-lactoglobulin, suggesting a change in conformation, but the minor troughs may have been obscured by instrumental noise. Unfortunately, the three-dimensional structure of

β-lactoglobulin is not known, although it behaves as a globular protein with strong self-association properties near its isoelectric point (McKenzie, 1971). Phosphorylation of ε-amines in β-lactoglobulin (e.g., Lys-60) also decreases ellipticity, but because dephosphorylation does not restore the original ellipticity, it is more likely that secondary and tertiary protein structures are being modified (Woo et al., 1982). Thus, the near-UV CD spectrum of proteins is an indicator of the extent of protein denaturation. The CD spectra of the modified β-lactoglobulins compared to those of the native protein and the protein completely randomized with Gdn·HCl indicated a more random structure as a result of amidation or esterification.

Since these modified proteins are more positively charged and are partially denatured, some of their functional properties in foods may also be altered. Systematic studies of alterations in protein functionality that result from structural changes may reveal structure–function relationships useful for increasing the utilization of whey proteins in foods.

ACKNOWLEDGMENT

We thank Professor W. W. Cleland of the Department of Biochemistry for use of the CD spectrometer.

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Received for review February 22, 1982. Revised manuscript received April 4, 1983. Accepted April 19, 1983. This research was supported by the College of Agricultural and Life Sciences and the Walter V. Price Cheese Research Institute, University of Wisconsin—Madison, and by grants from the Cooperative Research Service of the U.S. Department of Agriculture.

Physicochemical and Functional Properties of Positively Charged Derivatives of Bovine β-Lactoglobulin

Nina L. Mattarella[1] and Thomas Richardson*

Physicochemical and functional properties of positively charged amidated, ethyl-esterified, and methyl-esterified derivatives of bovine β-lactoglobulin were markedly different from those of β-lactoglobulin. Mean isoionic points for the proteins were 9.8, 8.7, and 9.5, respectively, for the derivatives compared to 5.2 for β-lactoglobulin. Blocking exposed carboxyl groups on β-lactoglobulin decreased the absorbance between 260 and 310 nm, increased the rate of tryptic hydrolysis, and decreased the rate of peptic hydrolysis. Methyl or ethyl ester groups were hydrolyzed only 7–14% when solutions of proteins were held at pH 10 for 24 h at 26 °C. Porcine liver esterase did not cleave ester bonds under conditions tested. Emulsion activities of modified proteins were less than that of β-lactoglobulin, but the stability of emulsions prepared with ethyl-esterified protein was greater, and this protein was 3.7-fold more concentrated at the oil/water interface than the other proteins. Binding of heptane by the proteins revealed that amidated β-lactoglobulin was most hydrophobic followed closely by ethyl-esterified β-lactoglobulin.

Bovine β-lactoglobulin, the major protein in cheese whey, is a well-characterized globular protein although its three-dimensional structure has not been defined with X-ray crystallography. Available knowledge on the structure of β-lactoglobulin coupled with its ready availability make it a useful protein to study structure–activity relationships of protein functionality in foods. Secondly, the utilization of surplus whey proteins in general, and β-lactoglobulin in particular, may be increased through a fundamental understanding of structural factors contributing to their functionality. Futhermore, useful nontoxic

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0021-8561/83/1431-0972$01.50/0